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SPERM AGGLUTINATION IN THE KEYHOLE LIMPET, MEGATHURA CRENULATA

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F. R. Lillie (1913) demonstrated that the sea water in which ripe sea-urchin eggs have been standing acquires the property of causing an agglutination of the spermatozoa of the same species. This iso-agglutination of sperm by egg water has since been reported in a number of animals, principally in the echinoderms, as the following list shows—

Echinoderms:

Arbacia punctulata (Lillie, 1913, 1914, 1919)
Strongylocentrotus purpuratus (Loeb, 1914; Lillie, 1921)
Strongylocentrotus franciscanus (Loeb, 1914; Lillie, 1921)
Asterias forbesii (Glaser, 1914; questioned by Just, 1930)
Echinarachnius parma (Just, 1915, 1919)
Paracentrotus lividus (Just, 1929)
Echinus microtuberculatus (Just, 1929)
Echinus esculentus (Carter, 1932)
Echinometra subangularis (Southwick, 1939)

Annelids:

Nereis limbata (Lillie, 1913)
Platynereis megalops (Just, 1915)

Mollusks:

Katharina tunicata (Sampson, 1922)

An interesting feature of the agglutination reaction, as described by Lillie, is that the agglutinates break up spontaneously. The clumps form very quickly after the addition of egg water, then after a few seconds or a few minutes, depending upon the concentration of egg water employed, reversal occurs. After complete agglutination and reversal the sperm cannot be re-agglutinated although they are alive and motile. The impression has been given, in the work on agglutination (see Lillie,

1919, p. 122; Lillie and Just, 1924, p. 489) that the spontaneous reversal is characteristic of iso-agglutination (i.e. by egg water of the same species), while non-reversal occurs only upon hetero-agglutination (i.e. by foreign egg water or other agents). In *Nereis,* however, Lillie himself has mentioned (1913, p. 552) that "the agglutinations are essentially permanent," and in *Katharina,* Sampson (1922) notes that some of the agglutinates reverse whereas others remain permanent.

In a recent note to *Science* it has been reported (Tyler and Fox, 1939) that the keyhole limpet, *Megathura crenulata,* exhibits an agglutination reaction which does not spontaneously reverse within the period during which the sperm remain viable. It has since been found that reversal may occur under certain conditions, particularly when excess sperm is employed. It was also reported that the form of the agglutinates is such as to indicate agglutination by the tails as well as by the heads of the spermatozoa, the picture being similar to that described by Sampson (1922) for *Katharina* sperm in egg water and by Henle, Henle and Chambers (1938) for bull sperm in anti-sera. In the present article a more detailed account of the agglutination reaction is presented together with some information concerning the source of the agglutinin and the specificity of the reaction. In a subsequent article, work on the chemical and physical properties and on the preparation of active concentrates will be reported.

Material and Methods

The giant keyhole limpet, *Megathura crenulata,* is obtained in fair numbers on the breakwaters and rocks at the entrance to Newport Bay and along the coast. They can be kept for a couple of months or more in running sea water aquaria. Sexually ripe individuals can be obtained at any season of the year, although they are relatively scarcer in the fall. The sexes cannot be distinguished externally. However, the animals can be "undressed" without injury and the gonads exposed to view. This is done by continuously stimulating, with a probe, the posterior edge of the mantle fold that covers the shell. The animal slowly retracts its mantle to beyond the edge of the shell which can then be raised slightly and the gonads examined through the transparent mantle that covers the viscera. The ovaries are dark green, the testes orange to creamy yellow in color.

The eggs and sperm are usually obtained by dissecting out the gonads, allowing them to shed in filtered sea water and straining through bolting cloth. Particularly in the case of the ovary the dissection involves injury to a small percentage (5 to 10 per cent) of the eggs. Occasionally the animals spawn in the aquaria. Sperm from the dis-

sected testes contains clumps of " immature " cells which are not present in spawned sperm. The behavior of these clumps will be described below. Agglutinin solutions from naturally spawned and from artificially removed eggs show no difference in action on the sperm. It would be desirable, however, particularly for the purpose of purifying the agglutinin, to obtain spawned eggs, since, in the limpet, injury to some of the eggs is almost unavoidable when the ovaries are dissected out. However, attempts to stimulate spawning have thus far been unsuccessful.

The sex products can be obtained in relatively large quantities. Individuals weighing about 300 grams have, when ripe, gonads weighing about 15 to 20 grams, of which more than three-fourths are mature eggs or sperm. The sperm can be kept in a viable condition for as long as a week by removing the testes " dry " to a stoppered vessel and storing at 4° C.

The egg water is obtained simply by removing the supernatant sea water in which eggs have been standing. With concentrated suspensions it is generally necessary to pack the eggs down in a centrifuge. Extraction by solutions other than sea water will be described in a subsequent article. It may be mentioned here that the agglutinin is obtained in highest titer by extraction with pH 3 sea water or isotonic NaCl.

The Agglutination Reaction

The agglutination reaction in the keyhole limpet is readily visible macroscopically. When a drop of egg water is mixed with a small amount of a sperm suspension (Fig. 1*a*), the latter very quickly assumes the mottled appearance shown in Fig. 1*b*. This change can occur in less than five seconds when highly concentrated solutions of the agglutinin and strong sperm suspensions are employed. It is due to the formation of small spherical clumps of sperm. The clumps enlarge by fusion with one another (Fig. 1*c*) and, in a sufficiently strong egg water, a single agglutinate, containing practically all of the sperm and resting on the bottom of the dish, results (Fig. 1*d*). This latter condition can be reached within five minutes after the addition of the egg water. The single agglutinate will form even if the dish is left undisturbed after the initial mixing. It is generally circular in outline. In strong egg water the periphery usually has a perfectly smooth appearance rather than the somewhat irregular form shown in Fig. 1*d*. The size of the agglutinate depends, of course, on the amount of sperm employed. It also depends on the strength of the egg water. As the egg water is diluted the compactness of the clump first decreases. Then the number

of initial agglutinates decreases and they fail to fuse into a single clump. Finally a dilution is reached at which no visible agglutination occurs. The time at which agglutination first becomes visible also varies with the strength of the egg water and the sperm suspension as will be shown below.

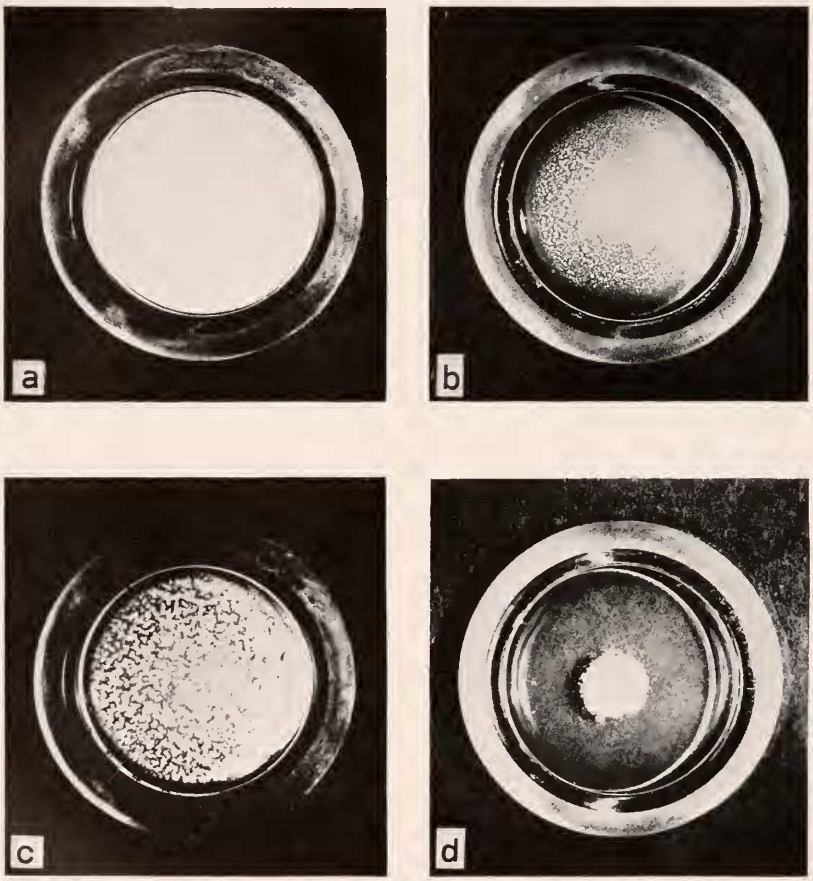

FIG. 1. Macroscopic appearance of the agglutination reaction in *Megathura*. Photographed in Syracuse dishes. × ⅔. *a*, Untreated sperm suspension (ca. 2 per cent); *b*, 15 seconds after addition of egg water; *c*, 30 seconds; *d*, 10 minutes.

The small agglutinates are typically spherical in shape. The larger ones are considerably flattened. The shape is similar to, although somewhat flatter than, that assumed by a drop of mercury in a dish of water.

The form of the agglutinates as seen under the microscope varies with the strength of the egg water employed and with the original con-

dition of the sperm. In the following account an egg water will be designated as strong if, when mixed with an equal volume of a one per cent sperm suspension, it gives an agglutination reaction that is macro-

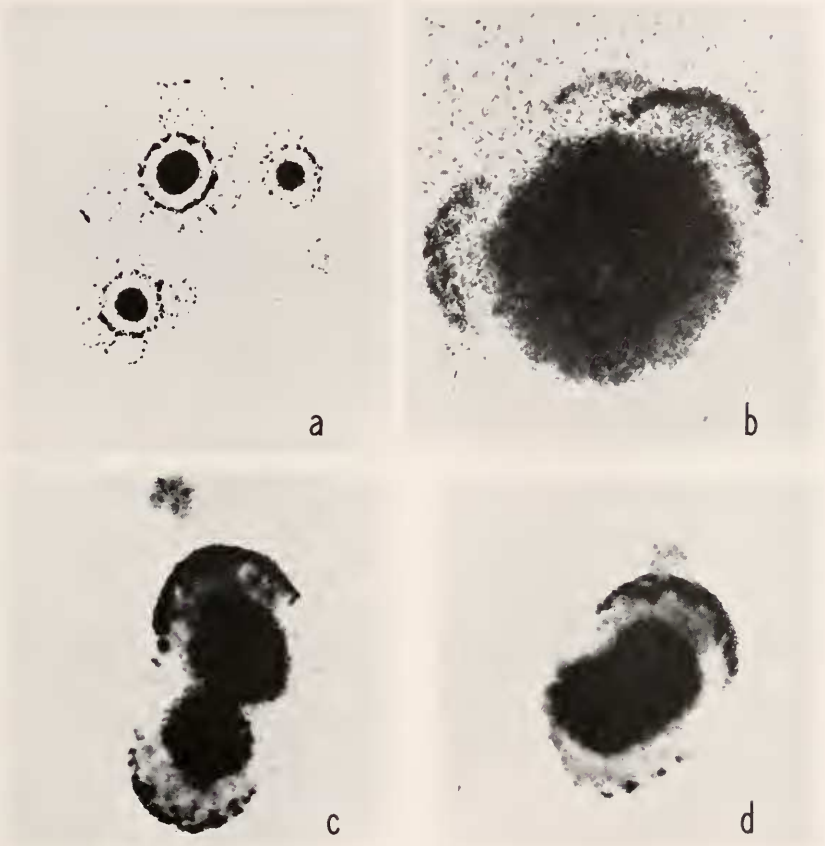

FIG. 2. Photomicrographs of sperm agglutinates of *Megathura*. *a*, Agglutinates formed in moderately strong egg water, showing spherical shell of sperm heads surrounding central mass of sperm. × 45. *b*, Agglutinate formed in strong egg water, showing shell segments derived mainly from sperm clumps of the type shown in Fig. 4*e*. × 150. *c* and *d*, Fusion of two agglutinates; *d* is taken 15 seconds after *c*. × 150.

scopically visible in 10 seconds or less; moderately strong if visible in 10 to 60 seconds and weak if visible in more than 60 seconds.

Figure 2*a* shows, under low magnification, the type of agglutinates obtained with moderately strong egg water. They are composed of a spherical shell of sperm heads attached by their tails to a central spheri-

cal mass of sperm. In the smallest agglutinates the central mass is lacking, only the ends of the tails occupying the center. This is shown in Figs. 3*a* and *b*. The sperm heads of the shell are united in small clusters. When strong egg water is employed, a distinct shell does not form. In Fig. 2*b* an agglutinate in strong egg water is shown under high power. This figure shows three segments of a shell attached by the tails to the main mass of sperm. These segments arise from clumps of sperm initially present in the untreated sperm suspension (Figs. 4*d* and *e*). When such clumps are absent in the original sperm suspension, no shell segments are seen on the agglutinates produced in strong egg water. The sperm at the periphery of such "shell-less" agglutinates are attached by their tails to the central mass. They are extremely active, the heads moving rapidly in all directions as far as the tails allow. Only occasionally does a spermatozoön break away from the clump. In the smallest agglutinates formed in strong egg water, the center is occupied by the fused tails and the heads are independently and very actively motile. These agglutinates resemble very much the tridimensional pinwheels described by Sampson (1922) for the isoagglutination of sperm of the black chiton, *Katharina tunicata*. The "shell-agglutinates" can be converted into the "shell-less" type simply by the addition of some highly concentrated egg water. This also greatly increases the activity of the spermatozoa.

The agglutinates enlarge by fusion with one another. Figures 2*c* and *d* show the fusion of two agglutinates. When a partial shell is present they generally unite in the region where the shell is lacking, as shown in the figures. The presence of nearly complete shells retards, but does not prevent fusion and a single shell always results after the union. The shell itself does not increase appreciably in thickness, nor in distance of separation from the central mass even after the fusion of a great many agglutinates that have nearly complete shells. This must mean that after repeated fusions some of the sperm from the shells are withdrawn into the central mass.

Head and Tail Agglutination

Henle, Henle and Chambers (1938) have described agglutination reactions of bull sperm to antisera prepared in the rabbit that resemble very much the reactions described here. According to these investigators the bull sperm agglutinate head to head and tail to tail. By separating heads and tails, by absorption of antisera with heads or tails, and by immunizing separately with the parts they obtain strong evidence for the existence of specific head and tail antigens and antibodies. In the keyhole limpet, too, head-to-head and tail-to-tail union of the sperm

can readily be found. However, the origin and behavior of such agglutinates indicate that only one kind of agglutinin is present in the original egg water.

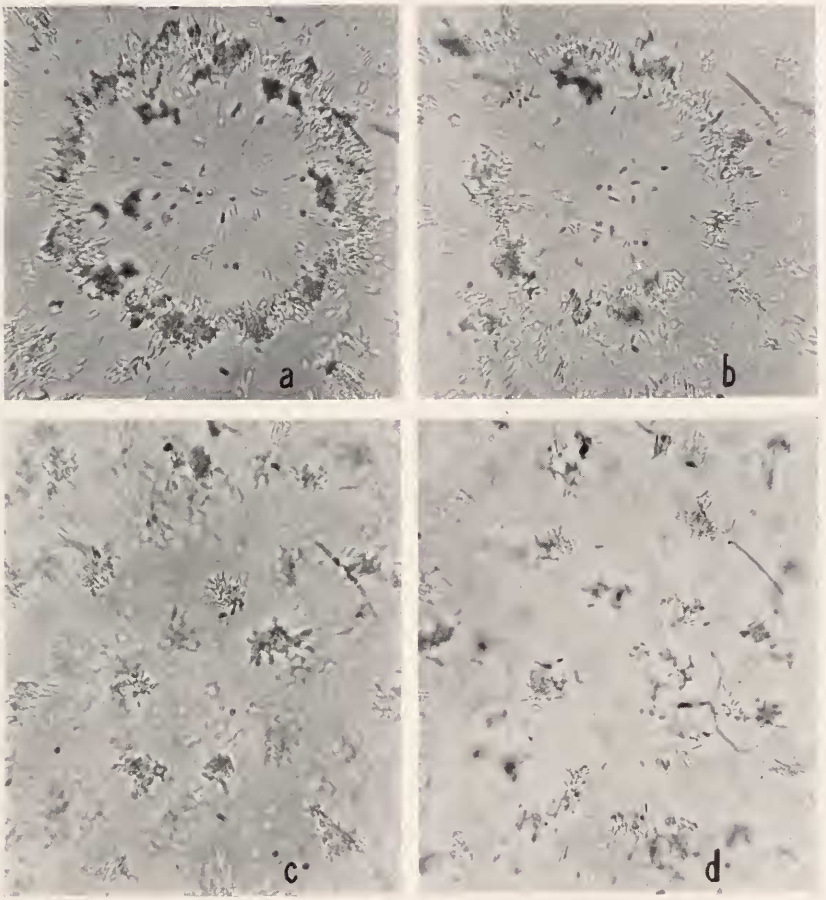

FIG. 3. Photomicrographs of sperm of *Megathura;* all × 300. *a* and *b*, Agglutinates formed in moderately strong egg water; taken before formation of central mass; shows tails agglutinated at center and heads clumped in groups (aggregated) at periphery. *c*, Agglutination in weak egg water; from each clump (aggregate) of sperm heads, bundles of tails (not distinctly visible) extend out in several directions and agglutinate with tails from other clumps. *d*, Aggregation reaction after dilution of sperm with sea water; the tails (indistinct) are not united with one another.

Two types of agglutinates showing both head-to-head and tail-to-tail union are found with keyhole limpet sperm. One type has been briefly described above (p. 164). It is obtained when a moderately strong egg

water is employed. In the shells of the agglutinates, there are seen small clusters of about 20 to 50 heads. These are particularly distinct in the small agglutinates shown in Figs. 3*a* and *b* that lack the central mass of sperm. The head clumping disperses readily upon mechanical agitation and soon reappears upon standing. Addition of concentrated egg water causes the head clumping to disappear, as was mentioned above. In weak egg water no typical shells are formed. Bundles of tails extend out in several directions from the small clumps of heads, the tail bundles of different clumps uniting by their ends. Figure 3*c* represents that type of agglutination. The tails, however, do not show up well in the photographs due presumably to the smaller number present in the bundles and their irregular distribution in three dimensions. When only sea water is added to a sperm suspension a similar clumping of heads but without union of tails occurs as shown in Fig. 3*d*. This kind of behavior of sperm was originally described by Lillie (1913) in *Nereis* and is termed by him aggregation. The aggregation phenomenon has been shown by Lillie (1914) to be due to the CO_2 production of the sperm. The aggregates readily disperse upon mechanical agitation. Lillie showed that it was necessary for the sperm to be active in order to aggregate. Thus dilution with sea water increases the activity of the sperm and results in aggregation. Within the aggregates, however, the sperm are inactive. It is evident that the aggregation phenomena will account for the head-to-head clumping observed in the weak or moderately strong egg water. The egg water causes greater activation of the sperm than does dilution with sea water. It also gives more extensive aggregation. In strong egg water the keyhole limpet sperm remain intensely active for many hours. It is perhaps understandable then that aggregation (head-to-head clumping) should fail to occur. However, even after the sperm have quieted down (i.e. at the periphery of the agglutinates) in strong egg water there is no aggregation. The discrepancy may perhaps be explained by an interpretation that is offered for the aggregation phenomenon in the discussion part of this paper.

The other type of head-to-head and tail-to-tail "agglutination" that is found owes its origin to the presence, in the untreated sperm suspension, of clumps of sperm in which the heads are united. Figures 4*d* and *e* show two such clumps. They are composed of groups of about 50 to 100 spermatozoa attached firmly by their heads and with their tails radiating out separately. Upon the addition of egg water the ends of the tails agglutinate and the clump assumes one of the forms shown in Figs. 4*a*, *b* and *c*. The parachute-shaped agglutinate shown in Fig. 4*a*

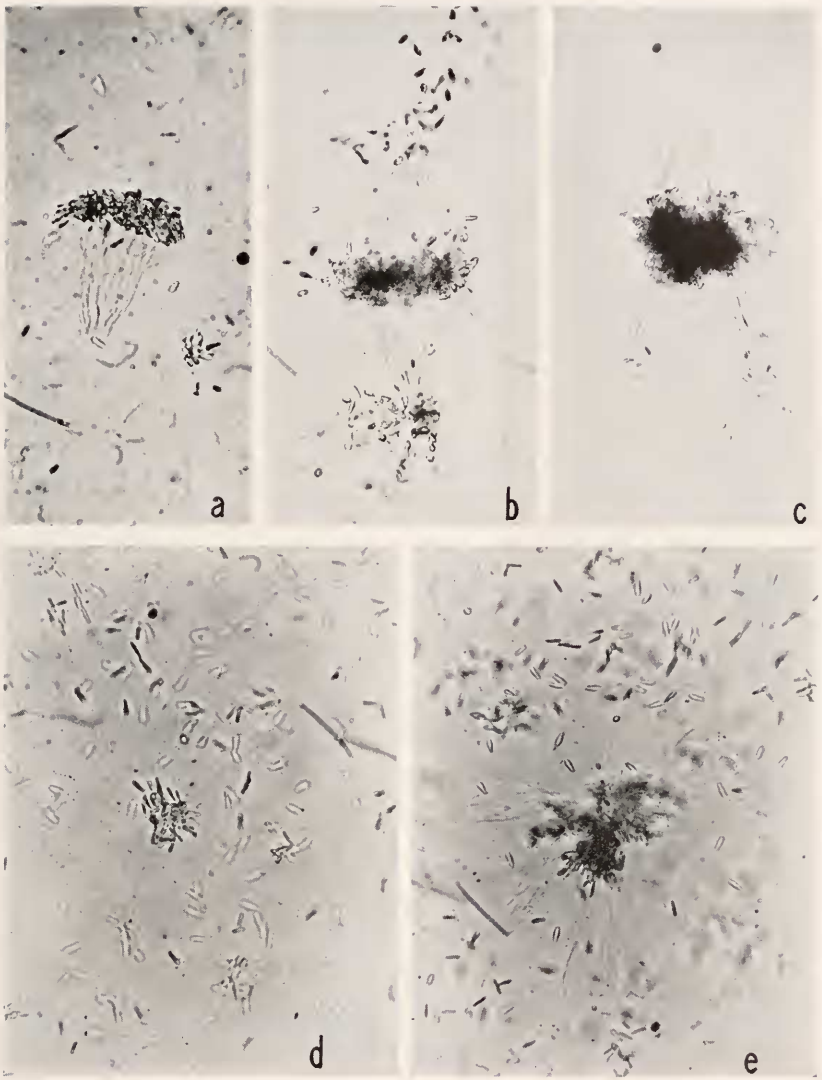

FIG. 4. Photomicrographs of sperm of *Megathura;* × 300; shows types of tail agglutination formed in: *a,* strong; *b,* moderately strong; *c,* weak egg water; from: *d* and *e,* "original" head clumps present in sperm suspension.

is obtained when strong egg water is added. The free tails come together on one side of the original clump of heads. With moderately strong egg water a great many of the agglutinates, that form from the original sperm clumps, have the spindle-shaped appearance shown in Fig. 4*b*. With weak egg water most of the agglutinates are of the

type, shown in Fig. 4*c*, in which the tails come together at several foci. In other words, with increase in dilution of the egg water there is an increase in the number of foci at which the ends of the tails unite. The behavior of these "original" clumps parallels then the reaction of separate sperm to various dilutions of egg water.

These "original" clumps are not found in naturally shed sperm. Quite likely they consist of somewhat immature sperm that exude from the tubules that happen to be cut upon dissecting out the testes. The clumps cannot be broken up very readily by shaking the sperm suspension. Even in strong egg water the heads fail to separate and, as was mentioned above (p. 164), these clumps form the partial shells such as are shown in Fig. 2*b*. When they are very numerous they may form nearly complete shells. The number of these original clumps obtained in a sperm suspension varies with the condition of the testis and the amount of damage done in dissection. In a sperm suspension obtained from a very ripe male in which the testis was removed with care and allowed to shed undisturbed, haematocytometer counts showed one clump to 12,000 free sperm. A suspension obtained by shaking fragments of a small (not fully ripe) testis in sea water showed approximately 250 clumps to 12,000 free sperm. As the sperm suspension is kept the number of clumps gradually diminishes. Thus the last-mentioned suspension showed after five days storage at 4° C. 45 clumps to 12,000 free sperm.

The above evidence shows, then, that there is only one kind of agglutinin, namely tail-agglutinin, present in egg water of the keyhole limpet. The head-to-head unions, that are observed, arise in one of the two ways described above, namely from the "original" sperm clumps or as a result of the aggregation phenomenon. In neither of these is it necessary to assume the presence of a separate head agglutinin in the egg water. This conclusion differs, then, from that reached by Henle, Henle and Chambers (1938). It does not, however, necessarily conflict, since their experiments were performed with immune sera (produced by injection of bull sperm into rabbit) whereas the present experiments concern natural agglutinins.

Rate and Duration

The time at which agglutination first becomes macroscopically visible can be used as a convenient measure of the rate of the reaction. The rate measured in this way varies with the concentration of egg water and of sperm suspension. While in general the rate decreases with decrease in egg water concentration, the relation is not one of direct

proportionality. Starting with a very strong egg water there is, on dilution, at first very little change in rate then a decrease roughly proportional to the dilution. With constant egg water concentrations, decrease in sperm concentration generally causes the rate to pass through a maximum. In addition, concentrated sperm suspensions may fail to give a visible reaction with certain egg water dilutions that do act on more dilute suspensions.

TABLE I

Time in seconds for agglutination to become macroscopically visible on mixing equal volumes of different concentrations of egg water and sperm suspension. Initial egg water = supernatant from a 10 per cent egg suspension, centrifuged after 2 hours. Initial sperm suspension = 30 per cent dry sperm = approx. 7.5×10^9 spermatozoa per cc. Temperature = 20° C.

rev = spontaneous reversal of agglutination within 24 hrs.
d = spermatozoa dead within 24 hours.

Egg Water Dilutions	Dilutions of Sperm Suspension							
	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
1	7	6	4	3	3	4	6 (*d*)	8 (*d*)
1/2	9	7	5	4	4	5	8	14 (*d*)
1/4	15	8	7	5	5	6	10	17 (*d*)
1/8	35 *rev*	14	10	7	7	10	15	20
1/16	105 *rev*	49 *rev*	21	14	13	17	28	30
1/32	—	—	140 *rev*	44	35	32	51	62
1/64	—	—	—	155 *rev*	190	150	140	110
1/128	—	—	—	—	—	420	300	260

These relations are shown in Table I, which contains the data of one of four similar experiments in which varying dilutions of egg water and sperm suspension were employed. As the data show, increasing the egg water concentrations for a given sperm suspension speeds up the reaction, but the increase in rate is greater with the dilute than with the concentrated egg waters. Decreasing the concentration of sperm for a given egg water at first speeds up the reaction, then retards it. Certain dilutions of egg water fail to give a reaction with concentrated sperm but do so with weaker suspensions. Thus $\frac{1}{32}$ egg water does not give a visible reaction with undiluted or $\frac{1}{2}$ diluted sperm; $\frac{1}{64}$ egg water fails with $\frac{1}{4}$ sperm, etc. This absence of a visible effect with certain ratios of the reactants is a familiar occurrence in serological reactions. It is known as the zone phenomenon (see Marrack, 1938; Zinnser, 1939). The non-

agglutinating zone shown in the lower left corner of Table I corresponds to that of antigen (sperm) excess. The zone of antibody (agglutinin) excess cannot be determined macroscopically since it involves too great a dilution of the sperm suspension. It has, however, been noted microscopically that very dilute sperm fail to agglutinate in strong egg water.

In serological reactions the ratio of antigen dilution to antibody dilution at the points of most rapid flocculation is essentially constant. Here the ratio shifts from 1:8 (or 1:16) in the strongest egg water to 1:1 in the weakest. A number of factors may contribute to this divergence from a constant ratio. Thus the activity of the spermatozoa increases with dilution and also with increased concentration of egg water; the pH varies slightly with the concentration of sperm due to CO_2 production; and the ease of determining the beginning of agglutination varies with the sperm concentration due to the differences in opacity of the different suspensions. In view of these various factors there is no point at present in attempting a further analysis in terms of the zone phenomenon. The data show, however, that it is important to take into account sperm as well as egg water concentration in assaying the agglutinin. It may further be noted that sperm suspensions from different animals and of different ages will vary considerably in their reaction times.

The duration of the agglutination also varies with the relative concentrations of sperm and agglutinin. In general, with strong egg water and moderately concentrated sperm suspensions, the agglutination persists until after the spermatozoa die and disintegrate. With weak egg water and concentrated sperm suspension the agglutination reverses spontaneously. In the data that are given in Table I the cases in which spontaneous reversal occurred within 24 hours are marked *rev.* In all the other cases the sperm were still agglutinated at that time. In certain cases marked (*d*) in Table I the spermatozoa were all dead in 24 hours. This was determined by noting whether or not the spermatozoa became motile upon dilution with sea water. As may be seen in the table, it is in the more dilute suspensions that the spermatozoa are found to be dead in 24 hours. It is, of course, well known that spermatozoa die sooner in dilute than in concentrated suspensions. The data show that, in addition, egg water shortens their life. This is undoubtedly due to the fact that egg water increases the activity of the spermatozoa.

While in most of the combinations listed in Table I, agglutination had not reversed in 24 hours, there was a tendency in that direction in that the clumps became less compact and irregular. This was more pronounced the higher the ratio of sperm concentration to egg water. If the spermatozoa in the clumps are dispersed mechanically at any time

while they are alive they will re-agglutinate. This occurs more rapidly the higher the ratio of egg water to sperm concentration. In those cases in which the agglutination reverses spontaneously, the time at which reversal occurs increases with increase in concentration of the egg water. It is difficult to obtain accurate data on the time of reversal as it is likewise difficult to obtain in those cases accurate times for the initial appearance of agglutination. This is due to the fact that an excess of sperm must be employed and, with dilution of the egg water, a smaller proportion is agglutinated. In the two cases of reversal listed in the first column of Table I, the approximate times are 5 hours for the $\frac{1}{16}$ egg water and 10 hours for the $\frac{1}{8}$ egg water. After spontaneous reversal, the addition of more egg water fails to cause re-agglutination or gives a weaker reaction than the control (untreated) sperm. In the latter instance the reaction is undoubtedly due to the presence of sperm that had not agglutinated initially.

In the sea urchin, Lillie (1913) showed that after spontaneous reversal the spermatozoa cannot be re-agglutinated. The present findings show that the reaction in the keyhole limpet is quite similar in that respect. But whereas in the sea urchin the agglutination lasts only a short time (up to about 15 minutes) and always reverses while the sperm are alive, in the keyhole limpet the reaction persists in most cases as long as the sperm are alive (24 to 48 hours) and only reverses in those mixtures where there is an excess of sperm.

Source of the Agglutinin

In the sea urchin, Lillie (1914, 1921) showed that the jelly layer surrounding the eggs contains the agglutinin in high titer. Loeb (1914, 1915) considered the agglutinin to be identical with the jelly and reported that it could not be obtained from eggs (*S. purpuratus*) in which the jelly had been removed by acid. Lillie, on the other hand, considered the agglutinin to be continuously produced by the egg. He reported its production by jellyless eggs of *Arbacia* and *Strongylocentrotus franciscanus,* and, although he confirmed Loeb's findings with *S. purpuratus,* he interpreted it to mean that the amount produced was too small to detect.

Repetition of the experiment of Lillie and Loeb on *S. purpuratus* again showed that after removal of the jelly no detectable amount of agglutinin is produced. It was also found that the acid sea water, which is employed to remove the jelly, extracts the agglutinin in very high titer and that this is not increased by allowing the eggs to stand in sea water for some time prior to extraction. One experiment illustrating this

point may be cited. A suspension of *S. purpuratus* eggs in sea water was divided into three parts to which acid (0.6 cc. of 1 N HCl per 100 cc. of sea water → pH 3.5) was added after they had stood 5 minutes, 2 hours and 10 hours respectively. Samples of the supernatant sea water before the addition of the acid and of the neutralized acid sea water were tested on freshly diluted sperm. They gave the following times for the duration of agglutination:

Age of egg suspension	5 minutes	2 hours	10 hours
Reaction before acidification	no aggl.	3 to 4 minutes	7 to 9 minutes
Reaction after acidification	15 to 16 minutes	15 to 17 minutes	14 to 16 minutes

While in ordinary sea water the agglutinin titer increases, the acid sea water removes no more agglutinin after 10 hours than at the start. After removal of the acid sea water no agglutinin could be obtained from the eggs even after 24 hours standing in sea water. In another sample of the same eggs, the sea water was removed after 10 hours and acid sea water added. The acid sea water gave in this case a reaction lasting 8 to 9 minutes, while the control gave, as before, 7 to 9-minute reactions. There is then no evidence for the secretion of agglutinin by the eggs of *S. purpuratus*. It must either all be present in the jelly at the start or must be the jelly itself. As is well known, the jelly slowly dissolves as the eggs stand in ordinary sea water and this would account for the increase in agglutinin titer of the supernatant.

The jelly of the sea-urchin egg can also be dissolved by means of another agent, namely the proteinase chymotrypsin. This enzyme in a concentration of 1 per cent dissolves the jelly within about 15 minutes. At the same time it completely inactivates the agglutinin. After the treatment no agglutinin production could be detected. Again it appears that the agglutinin is the jelly or something intimately associated with it.

In the keyhole limpet the evidence points in the same direction. The jelly of these eggs dissolves much more slowly in acid sea water than does that of sea-urchin eggs, but by centrifuging in the acid solution jellyless eggs can readily be obtained. These when allowed to stand in sea water produce no detectable agglutinin. In the acid sea water the agglutinin is obtained in very high titer. The same type of experiment as described above for the sea urchin was performed on the keyhole limpet eggs, with the following results, for the time at which visible agglutination appears on testing the solutions.

Age of egg suspension	5 minutes	1 hour	2 hours
Reaction before acidification	4 to 6 minutes	30 seconds	12 seconds
Reaction after acidification	7 to 8 seconds	7 to 8 seconds	7 to 8 seconds

As before, the agglutinin titer (the time for visible agglutination decreases with increase in agglutinin concentration as shown in Table I) of the supernatant sea water increases with the time that the eggs remain in contact with it. There is, however, no corresponding increase in the amount of agglutinin that can be obtained after adding the acid. This remains essentially constant showing again no production of agglutinin by the eggs.

While the above evidence locates the agglutinin in the jelly layer surrounding the egg, it does not identify it with the jelly. To determine this point it would be necessary to know how many substances comprise the jelly. It seems unlikely that more than one is involved, but in the absence of direct information it may only be concluded at present that the agglutinin is a component of the jelly layer.

Specificity

Lillie (1919) and Just (1930) have shown that sperm agglutination is both tissue- and species-specific. In certain instances (e.g. *Arbacia* egg water or blood on *Nereis* or *Echinarachnius* sperm) cross reactions were obtained. It was noted in such cases that the reaction fails to reverse spontaneously and the term hetero-agglutination was used to designate the cross reactions. Since in the keyhole limpet the iso-agglutination also fails to reverse spontaneously under most conditions and resembles in this respect hetero-agglutination, it was of interest to examine the specificity of the reaction.

Tests were made with the blood and with sea water extracts of the foot, mantle and viscera of both male and female keyhole limpets. The extracts were prepared by washing the fresh tissues, by grinding them and by first freezing at —70° C. in sea water. In no case was agglutination of sperm obtained.

The egg waters of several different animals were tested on keyhole limpet sperm, and at the same time egg water of the keyhole limpet was tested on the foreign sperm. The animals examined were *Haliotis cracherodii* (abalone), *Astraea undosa* (top shell), *Lottia gigantea* (limpet), *Ischnochiton magdalenensis* (chiton), *Urechis caupo* (gephyrean worm), *Strongylocentrotus purpuratus* (sea urchin), *Dendraster excentricus* (sand dollar) and *Patiria miniata* (starfish). None of these showed either agglutination of keyhole limpet sperm with their egg water or agglutination of their sperm with keyhole limpet egg water. Of these animals the first two belong to the same division (rhipidoglossa) as the keyhole limpet. No animals belonging to the same family were available for testing. It would, of course, be of considerable interest to

examine more closely related animals in order to determine the extent to which the reaction is specific. The present results make it clear, however, that although there may be a superficial resemblance to what has been described as hetero-agglutination, the reaction in the keyhole limpet is tissue specific and at least to some extent species specific.

Discussion

As the evidence presented in the first part of this paper shows, there is present in egg water of the keyhole limpet only one kind of agglutinin, namely that for the tails of the sperm. The clumping of heads that occurs in all but the strongest egg water is evidently an aggregation phenomena. Aggregation, as Lillie (1913) first noted, occurs upon dilution of a sperm suspension. The addition of egg water leads to aggregation presumably for the same reason that dilution does, namely as a result of the increased activity of the spermatozoa.

Lillie showed that the aggregation reaction can be produced by the addition of CO_2 to the sperm suspension, but not if the CO_2 tension is too high, and that it fails to occur in sperm suspensions to which alkali has been added. He (1919, p. 103) interpreted the reaction as a chemotactic response to CO_2. It is, however, difficult to see how the necessary CO_2 gradient would be produced and maintained in a suspension of actively moving spermatozoa. Furthermore the sperm appear to be actually stuck to one another in the aggregates.

In view of these considerations, the following alternative hypothesis may be suggested for the mechanism of the aggregation reaction. The increased CO_2 production resulting from the increased activity of the sperm lowers the pH of the sea water and thereby produces some change on the surface of the sperm head that enables them to stick to one another when they meet. This change might be regarded as a general increase in stickiness. The sperm do not, however, show an increased tendency to stick to any object but rather only to one another. It seems preferable, then, to consider that the slight increase in acidity causes a partial dissociation of some surface material (S) of the sperm from the underlying substance (U) with which it is in loose combination. This would produce areas on the sperm head where U is exposed and capable of uniting with S on other sperm. In this manner clumps of several sperm heads could be formed. To explain the failure of aggregation to occur in strong egg water (p. 166), it would be necessary to assume that the greater activity of the spermatozoa caused a sufficient lowering of the pH to give complete dissociation.

The view presented here represents an extension of the lattice theory of agglutination (Heidelberger, 1938; Marrack, 1938). The agglutinin

here is assumed to be the surface material, *S*, initially present on the head of the sperm. The aggregation phenomenon is, then, regarded as an auto-agglutination reaction. It is attributed to a partial dissociation of the agglutinin occasioned by change in pH (or perhaps other factors as well) and combination of the underlying material with agglutinin present on other spermatozoa. The recent results of Southwick (1939*b*) with *Chiton* sperm could be interpreted on this basis. Auto-agglutination has been often described in bacteria and blood cells, but as far as the author is aware, no interpretation of this kind has been offered for the phenomena. To test the hypothesis it would be necessary to obtain from a particular kind of cell a substance capable of agglutinating cells of the same kind. We have not succeeded as yet in doing this with the sperm. With eggs of the sea urchin, however, we have been able to obtain evidence of this sort. That is, a substance can be extracted from the eggs that is capable of agglutinating eggs. This work will be reported in a subsequent article. It is mentioned here, however, as support for the interpretation suggested for the aggregation (auto-agglutination) reaction in sperm.

Heidelberger and Kabat (1936) report an experiment which, I believe, further supports this view. They " sensitized " bacteria (Pneumococcus I M) by adding excess agglutinin, and resuspended the coated cells in fresh saline. The addition of untreated cells of the same type causes the entire mass of cells to agglutinate. This does not occur when cells of other types are added.

In regard to the spontaneous reversal of agglutination, the keyhole limpet reaction evidently differs only in a quantitative manner from that in the sea-urchin. Thus under most conditions the agglutination in the keyhole limpet persists until the sperm are dead. Some further observations may be mentioned here that correlate with this difference in the two forms. A substance (anti-agglutinin) has been extracted from sea-urchin sperm (Frank, 1939; Southwick, 1939*a*) and from keyhole limpet sperm (Tyler, 1939) which has the property of neutralizing the agglutinin of eggs of the same species. When solutions of agglutinin and anti-agglutinin are mixed a precipitate is formed. In the sea urchin this occurs within about 2 to 10 minutes depending upon the concentrations. In the keyhole limpet the precipitate does not form until after 20 or more hours. Neutralization of the agglutinin, however, occurs almost immediately after addition of the anti-agglutinin in both species. The time of precipitate formation corresponds roughly to the time at which agglutination reverses in the two forms. It appears, then, that reversal is due to some secondary change in the compound formed by the combination of agglutinin with anti-agglutinin on the sperm, and that this

secondary change is analogous to the precipitate formation observed in the test tube on mixing solutions of agglutinin and anti-agglutinin.

In the sea urchin Lillie (*op. cit.*) showed that after spontaneous reversal the spermatozoa cannot be re-agglutinated. The same is true for the keyhole limpet as was noted above. Lillie (1913, p. 558) also noted a decrease in the fertilizing power of sperm suspensions after agglutination and reversal. I have confirmed and extended this with the sea urchin as well as with the keyhole limpet. These results, the details of which will appear in a later publication, show that sperm are no longer capable of fertilization although they are still highly motile after reversal of agglutination. It seems likely that the loss of the capacity for agglutination and for fertilization is due to the secondary change mentioned above. This would involve a change in the nature of the "reacting" surface of the spermatozoa either by removal of the reactive material, as Lillie proposed, or by the presence of altered agglutinin on the surface.

In the account of the agglutination reaction given in the first section of this paper (p. 162) it was pointed out that the form of the agglutinates is that of drops of a heavy liquid immiscible with water. Furthermore, separate agglutinates fuse with one another in very much the same manner as do liquid drops. These considerations lead, then, to the view that the initial reaction of agglutinin with the reactive material (anti-agglutinin) on the sperm causes the agglutinin to become insoluble in sea water and to separate out as a liquid. If such a change occurred in the agglutination reaction, one might expect to see it in the precipitin reaction; that is, on mixing solutions of agglutinin and anti-agglutinin. However, although there is an immediate neutralization of the agglutinin, no visible change occurs until some time later when the precipitate forms. No separation into two layers, as might be expected with two immiscible liquids, has as yet been seen. This does not necessarily rule out the postulated change since the conditions of the precipitin reaction might be such as to form very fine emulsions, whereas in the agglutination reaction the liquid might separate out from the sea water more readily due to the activity of the sperm, or the fact that the anti-agglutinin (on the sperm) is for the most part not in solution, or to other possible factors. Thus, while it seems almost essential, in order to interpret the form and behavior of the agglutinates, to assume that the agglutinin becomes an insoluble liquid, there is at present no direct evidence for or against that view.

Summary

1. Egg water of the keyhole limpet, *Megathura crenulata,* causes a strong agglutination of the sperm of this species. In contrast to the rapid spontaneous reversal that occurs in the sea urchin, the reaction here is relatively irreversible, the agglutinates persisting in most cases until after the death of the sperm. Spontaneous reversal occurs when an excess of sperm is employed.

2. When anti-agglutinin (sperm extract) is added to agglutinin (egg water) the latter is immediately neutralized and a precipitate later appears. The precipitate appears very much later with the keyhole limpet substances than with those of the sea urchin, the time corresponding roughly to that at which reversal of agglutination occurs in these two forms.

3. With varying concentrations of agglutinin and sperm suspension the occurrence and rate of agglutination behaves in a manner similar to that described as the zone phenomena in serological reactions.

4. The sperm agglutinate by their tails and under certain conditions by their heads as well. But the evidence shows only tail agglutinin to be present in the egg water, the head clumping being ascribed to the aggregation reaction which the sperm exhibit on dilution with sea water. The aggregation reaction is considered as an auto-agglutination and a new interpretation for this is offered based on the partial dissociation of a substance from surface of the sperm head as a result of change in pH or other conditions.

5. The agglutinin is shown to be a component of the jelly layer of the egg. No evidence of its continuous production by the egg was obtained in the sea urchin or in the keyhole limpet.

6. Although the reaction bears a superficial resemblance to what has been described as hetero-agglutination, it is found to exhibit both tissue and species specificity.

7. The agglutinates have the form and behavior of liquid drops. It is suggested that this is due to the agglutinin forming an insoluble liquid upon reaction with the spermatozoa.

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